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Integration Is Essential for Efficient Gene Expression of Human Immunodeficiency Virus Type 1

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A mutant of human immunodeficiency virus type 1 which carries a frameshift insertion in the integrase/endonuclease region of *pol* gene was constructed in vitro. Upon transfection into cells, although this mutant exhibited a normal phenotype with respect to expression of *gag*, *pol*, and *env* genes and to generation of progeny virions, no replication-competent virus in CD4-positive cells emerged. An assay for the single-step replication of a defective viral genome dependent on *trans* complementation by *rev* protein was established and used to monitor the early phase of viral infection process. Viral clones with a mutation in the *vif*, *vpr*, or *vpu* gene displayed no abnormality in the early phase. In contrast, the integrase mutant did not direct a marker gene expression after infection. Together with an observation that the mutant lacked the ability to integrate, these results indicated that the integration was required for efficient viral gene expression and productive infection of human immunodeficiency virus type 1.

The replication cycle of retroviruses consists of two major phases (4, 29). The first half of the cycle is directed toward the integration of a complete DNA copy of viral genes into host cell DNA. Shortly after entry into susceptible cells, the viral single-stranded RNA genome is converted into a linear double-stranded DNA. The viral DNA intermediate then migrates to the cell nucleus and is covalently integrated into a host chromosome. The second phase begins with the expression of the genes encoded in the provirus and ends with the production of infectious progeny viral particles. The two events in the early phase of the replication cycle, reverse transcription and integration, distinguish retroviruses from other classes of virus. These two processes are mediated by the two enzymes (reverse transcriptase and integrase) encoded by a virus single open reading frame, *pol* (5, 7).

Integration of a DNA copy of the viral genome into host cellular DNA is an essential step in the life cycle of most, if not all, retroviruses (4, 12, 29). Integration appears, moreover, to be important for the transcription of viral DNA into new copies of the viral genome and mRNAs that encode viral proteins (4, 12, 29). Integration-defective mutants of retroviruses are able to produce a DNA copy of the viral genome, but the replication of the mutants in the target cells is almost negligible (6, 14, 19, 20, 24). There are some reports on the functional role of the integrase of human immunodeficiency virus type 1 (HIV-1) in the replication cycle. Viral clones carrying a mutation in the integrase region of *pol* gene were unable to integrate (25) and were replication defective (2, 3, 12, 25). Surprisingly, in one article (25), the unintegrated HIV-1 DNA was reported to serve as an efficient template for HIV-1 antigen production. Because the integrase of HIV-1 is very likely to function in the early replication phase as do those of the other retroviruses, this result might represent a unique property of the HIV-1 integrase.

In this communication, an integration-deficient mutant of HIV-1 was constructed and analyzed in detail in several transfection and infection experiments. We demonstrate here that the HIV-1 integrase functions in the early replication phase and that integration is important for viral gene expression.

MATERIALS AND METHODS

Cell culture and DNA transfection. A human colon carcinoma cell line, SW480 (1), and a monkey kidney cell line, COS-1 (9), were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. $CD4^+$ human T-cell leukemic cell lines, A3.01 (8) and H9 (21), were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal calf serum. For transfection, uncleaved plasmid DNA was introduced into adherent (SW480 and COS-1) and nonadherent (A3.01 and H9) cells by the calcium-phosphate coprecipitation (11, 30) and modified DEAE-dextran (27) methods, respectively.

Infection. The infectivity of progeny virions produced in the transfected SW480 cells was assayed in A3.01 cells. Culture supernatants were filtered (pore size, 0.45 μm), and appropriate volumes were added to 10^6 cells as previously described (8).

RT assays. Virion-associated reverse transcriptase (RT) activity was measured as described previously (31). For quantitation, spots on DE81 paper were cut out, and RT activity was determined by scintillation counting.

CAT assays. The chloramphenicol acetyltransferase (CAT) assay has been previously described (10). CAT levels were assayed in equivalent amounts of cell lysates from transfected SW480 cells and infected A3.01 cells.

DNA constructs. An infectious proviral clone of HIV-1, pNL-432, and its mutants, designated pNL-Af1 (*pol*/integrase mutant), pNL-Nd (*vif* mutant), pNL-Af2 (*vpr* mutant), and pNL-Ss (*vpu* mutant), have been previously described (1, 2, 17). pNL-nCAT and the mutant clones of pNL-nCAT were constructed from pNL-432 (1) and its mutant clones,

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which have been previously described (2, 17). To make a series of pNL-nCAT constructs, the CAT coding sequence of pSV2CAT (784-bp *Hin*dIII-*Sau*3AI fragment) (23) was inserted into the *Xho*I (nucleotide [nt] 8887) and *Kpn*I (nt 9005) sites in the *nef* coding region of parental clones, and the *rev* coding sequence was inactivated by insertion of an 8-bp *Xba*I linker (Toyobo Inc., Osaka, Japan) into the *Bam*HI site (nt 8465) (see Fig. 2). pNL-nCAT-Nh was generated from pNL-nCAT by digestion with the *Nhe*I restriction enzyme, by blunt ending with T4 DNA polymerase, and by resealing with T4 DNA ligase. Construction of pNL-gCAT and prev1 has been described previously (22, 23).

EM. Transfected SW480 cells were processed for electron microscopy (EM) by the standard method (2). Briefly, the cells were prefixed with Karnovsky's fixative (1.0% paraformaldehyde–3.0% glutaraldehyde in 0.2 M cacodylate buffer [pH 7.4]) at 4°C for 1 h, postfixed with 1% osmium tetroxide at 4°C for 1 h, and then dehydrated in a graded ethanol series and embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate solution, and observed under a Hitachi HU12A electron microscope.

Extraction of extrachromosomal and integrated virus DNA. Extrachromosomal virus DNA in the infected cells was extracted at 10 h postinfection by the method of Hirt (15). High-molecular-weight cellular genomic DNA was extracted from cells 2 days after infection by a standard proteinase K-phenol spooling method. RNA was removed from DNA solution by digestion with RNase H and RNase T_1 (Sigma Chemical Co., St. Louis, Mo.).

PCR analysis of viral DNA. DNA samples were pretreated with *Dpn*I restriction endonuclease (New England Biolabs, Inc., Mass. USA) to remove plasmid DNAs potentially contaminated. Polymerase chain reaction (PCR) amplification was performed with two primers, V3DF (nt 6990 to 7011 of pNL-432) and SK69 (nt 7927 to 2907 of pNL-432) (18). Samples (2 μg of DNA) were subjected to 24 cycles of PCR in a 50-μl reaction mixture. Composition of reaction mixture was as the supplier suggested for *Tth* DNA polymerase (Toyobo Inc.). Each cycle consisted of 1 min of denaturation (93°C), 2 min of annealing (57°C), and 3 min of extension (72°C). Amplified products were run through a 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, Md.) and analyzed by Southern blotting hybridization. The ^{32}P-labeled *Sal*I (nt 5785)-*Xho*I (nt 8887) fragment produced by the random primer DNA labeling kit (Takara Shuzo Co., Ltd., Kyoto, Japan) was used as a probe.

Nucleotide sequence accession number. Nucleotide sequence data of pNL-432 are from GenBank data base accession number M19921.

RESULTS

Transfection analysis of HIV-1 integrase mutant. A proviral DNA clone of HIV-1, designated pNL-Af1, which carries a frameshift insertion within the integrase region of *pol* gene (2) was transfected into CD4-negative SW480 cells (1) and examined for its expression. We previously reported that the mutant expressed viral structural proteins and produced progeny virions normally in transfected SW480 cells, as judged by Western immunoblotting analysis and RT assay, respectively (2). As shown in Fig. 1A, this clone could also direct the synthesis of progeny virions morphologically similar to wild-type (wt) particles upon transfection, and no significant difference between the amounts of progenies

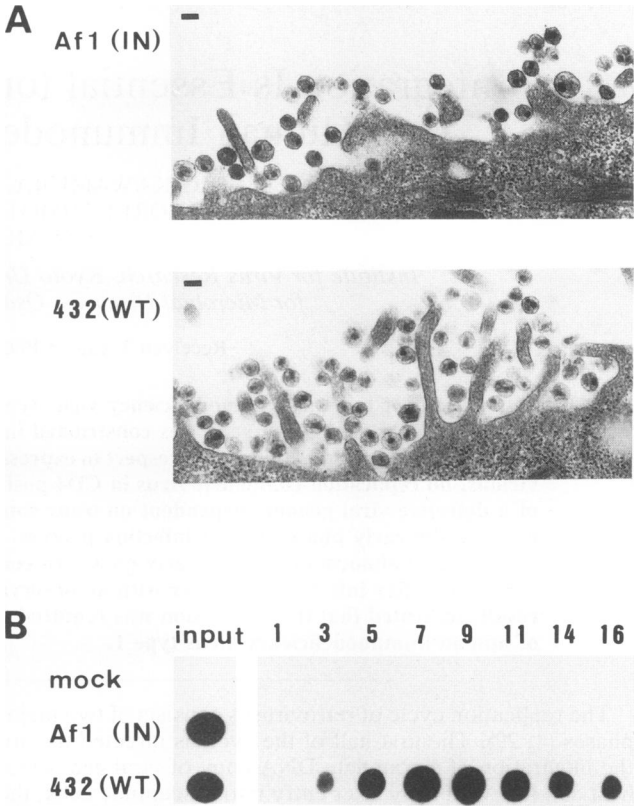

FIG. 1. Characterization of an integrase mutant of HIV-1. SW480 cells were transfected with pNL-432 (HIV-1 wt) (1) or pNL-Af1 (HIV-1 integrase mutant) (2), and 48 h later, samples were prepared for EM (A) and for infection (B). (A) EM picture of virions produced by transfected SW480 cells. Af1 (IN), virus from pNL-Af1; 432(WT), virus from pNL-432. Bar = 100 nm. (B) Infection of CD4-positive cells with virus. For infection, A3.01 cells (10^6) were infected with 10^5 RT units of cell-free virus obtained from transfected SW480 cells (input). RT production in the culture fluids of infected cells was monitored at intervals. Virus designation is the same as presented in panel A. Values indicate days after infection.

produced by wt and mutant clones was observed. The NL-Af1 virus obtained from transfection, however, was unable to establish a productive infection in CD4-positive A3.01 cells. RT activity was not detected in the cells inoculated with the NL-Af1 on any days postinfection (Fig. 1B). These results were consistent with those presented in previous reports, including ours (2, 3, 12, 25), and suggested that the viral integrase protein plays an important functional role in the early phase of viral replication cycle, at least in the SW480-A3.01 system. To examine the early replication phase further, a new assay system to quantitate viral infectivity (from virus entry to transcription) similar to those previously described (13, 28) was established. This system utilized replication-defective *rev*-minus proviral clones containing the CAT gene in the *nef* region (Fig. 2) and was dependent on *trans* complementation for production of infectious vector virus (Fig. 3). When the *rev* was supplied in *trans*, infectious viruses, which carry the CAT gene and are capable of undergoing one round of replication, would be generated (Fig. 3). Infection of cells with the virus and the CAT activity obtained would provide a measure of the efficiency with which the early infection phase of the virus

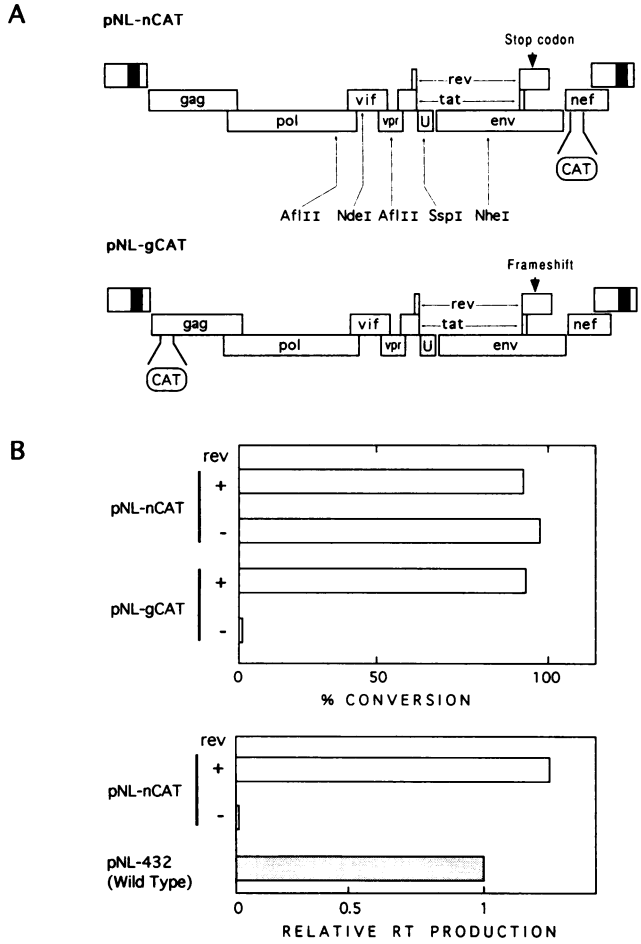

FIG. 2. Replication-defective proviral clones carrying a CAT gene used for a single-round infection assay. (A) Genome structures of pNL-nCAT and pNL-gCAT. pNL-nCAT constructed in this study contain a CAT gene in the *nef* gene region and an *Xba*I linker insertion in the 2nd coding exon of the *rev* gene. As a result of the linker insertion, the *rev* protein would stop at the 60th amino acid, and the *env* protein would have a 4-amino-acid insertion at the site. The mutant clones of pNL-nCAT, designated pNL-nCAT-Af1, pNL-nCAT-Nd, pNL-nCAT-Af2, pNL-nCAT-Ss, and pNL-nCAT-Nh, have frameshift mutations at the restriction enzyme sites indicated. Generation and characterization of pNL-gCAT were described previously (22). (B) *rev* dependency of marker gene expression. pNL-nCAT and pNL-gCAT (5 μg) were transfected into SW480 cells with (+) or without (−) *rev* expression plasmid prev1 (10 μg). Two days after transfection, CAT activity in the cell lysates (upper graph) and RT production into culture supernatants relative to that by pNL-432 (5 μg) (lower graph) were determined. RT counts produced by pNL-432 were 2,885 cpm.

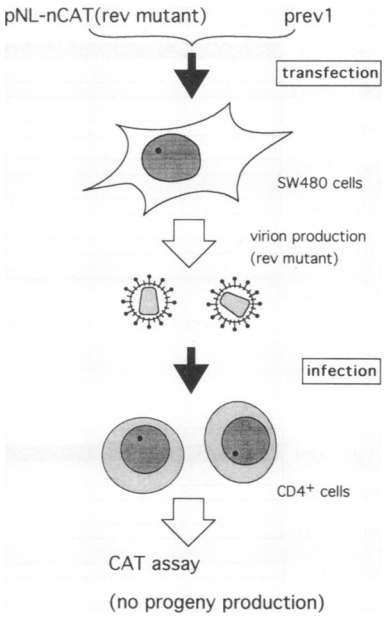

FIG. 3. A single-step infection assay. Virions recovered from SW480 cells cotransfected with replication-defective pNL-nCAT constructs (Fig. 2) and *rev* expression vector prev1 (22, 23) are able to undergo one round of replication but not multiple cycles of infection in CD4-positive cells. Early phase of viral replication can be monitored by the highly sensitive CAT assay.

proceeds. As shown in Fig. 2B, while the pNL-nCAT construct (wt) for this assay expressed CAT activity independently of the presence or absence of the *rev* protein within transfected SW480 cells, its progeny production into a culture supernatant was *rev* dependent. A series of pNL-nCAT mutant constructs (Fig. 2A), which carry the same alteration in either the *pol* (integrase) or the other genes (*vif*, *vpr*, *vpu*, and *env* [gp120]) as previously reported (2, 17) in addition to the *rev* gene mutation, were transfected with prev1 (HIV-1 *rev* expression plasmid [22, 23]) into SW480 cells to determine the effect of the mutation on the expression of the marker CAT gene and the progeny production. As is clear in Fig. 4, none of the mutations affected CAT and virus production, which is in agreement with our previous data (2, 17). To determine whether these results represent a cell-specific phenomenon, several cell lines, including CD4-positive A3.01 and H9, were transfected with wt or the integrase-minus mutant clone and monitored for RT production. As shown in Fig. 5, no significant difference, again, in the level of progeny production in any of the cell lines tested was observed. None of the progeny viruses produced in transfected COS-1, A3.01, and H9 cells were infectious (data not shown).

Of particular note is that the integrase mutant exhibited the wt phenotype in all transfection experiments described above. Because transfection of a viral DNA clone into cells would bypass the early phase of viral replication (adsorption, penetration, uncoating, and reverse transcription), these results are certainly consistent with the requirement of the integrase protein in the early infection stage.

Analysis of early infection phase of the mutant clones. The effect of mutations in the viral genes on the early stage of virus replication was studied by the system described in Fig. 3. Cell-free virus samples, collected on day 2 posttransfection into SW480 cells, were inoculated into CD4-positive A3.01 cells, and 2 days later, cell lysates were prepared for CAT assay. Because the gp120^{env} plays a critical role for the first step in the infection cycle, the *env* mutant carrying a frameshift insertion in the upstream region of CD4-binding domain (Fig. 2A) was used as a negative control for this experiment. As shown in Fig. 6, approximately 100-fold increases in A3.01 cell CAT activity were observed between the positive (wt) and negative (*env*) control samples. In agreement with the finding that the *vpu* protein is required in the late infection phase (maturation/release) (16), the muta-

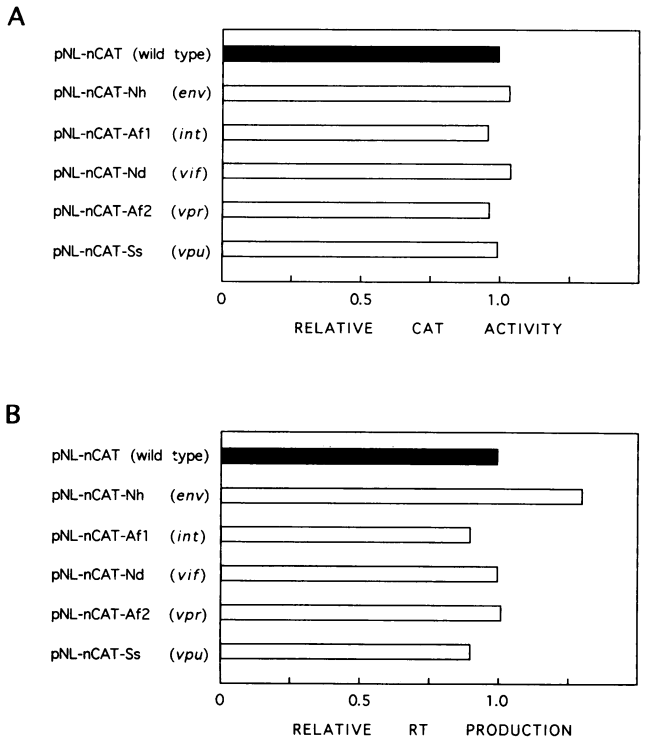

FIG. 4. Expression of marker gene by transfection of a series of pNL-nCAT constructs. The provirus-CAT clones (Fig. 2) and prev1 (*rev* expression vector) were cotransfected into SW480 cells, and 48 h later, CAT activity in the cell lysates (A) and RT activity in the culture supernatants (B) were determined. Relative activity is shown (that of wt pNL-nCAT = 1). CAT and RT activities expressed by the cells cotransfected with pNL-nCAT and prev1 (black bars) were 24.8% (conversion ratio) and 3,124 cpm, respectively. Mutant designations and mutated genes (in parentheses) are indicated.

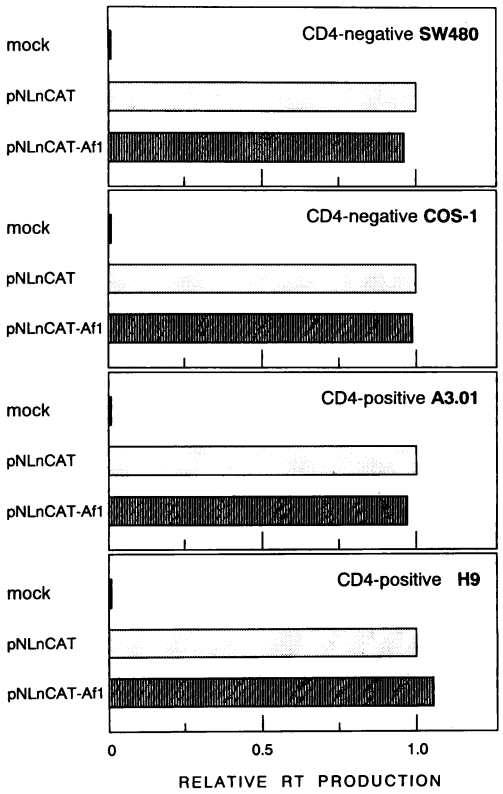

FIG. 5. Progeny production in various cell lines transfected with the integrase mutant. Four cell lines were transfected with a pair of pNL-nCAT (wt) plus prev1 (*rev* supplier) or pNL-nCAT-Af1 (integrase mutant) plus prev1, and 48 h later, RT activity in the culture supernatants was determined. Relative RT titer is shown. RT counts produced by various cell lines cotransfected with pNL-nCAT and prev1 were 3,285 cpm (SW480), 1,898 cpm (COS-1), 1,825 cpm (A3.01), and 965 cpm (H9).

tion in *vpu* had no effect on the production of CAT. A similar level of CAT activity was also observed in A3.01 cells infected with the *vif* or *vpr* mutant viruses. In contrast, no significant increase in A3.01 CAT activity (relative to the activity of the negative control) was detected when the integrase mutant was used as an inoculum. The experiments were repeated three times with essentially the same results. Furthermore, the results were reproduced in two other T-cell lines (H9 and M8166) and a monocytic cell line, HL60 (data not shown).

To determine whether viral DNA is synthesized and integrated into a chromosomal DNA of A3.01 cells infected with various virus clones, PCR analysis was performed (Fig. 7). Hirt's DNA (15) and high-molecular-weight genomic DNA were extracted from the infected cells at the appropriate time. The *env* sequence in the samples was amplified by the PCR procedure and probed by a ^{32}P-labeled viral DNA fragment. While a 937-bp target of the HIV-1 *env* sequence could be amplified in both Hirt and genomic fractions of A3.01 cells infected with wt virus, the *env* sequence was detected in neither fraction of the cells infected with the *env* mutant virus, as expected (Fig. 7B). Consistent with the data in Fig. 6, mutant viruses of the *vif*, *vpr*, and *vpu* genes were able to synthesize viral DNA and integrate into host cell DNA. Although HIV-1 DNA was produced normally in A3.01 cells infected with the integrase mutant, no proviral DNA integrated into cellular DNA was found. This result demonstrated the integration-defective property of our integrase mutant.

DISCUSSION

The results presented in this study demonstrate that unintegrated viral DNA is an extremely inefficient template for HIV-1 expression in the natural infection system and that integration mediated by viral integrase protein is essential for HIV-1 productive infection. An integration-defective (Fig. 7) integrase mutant constructed in vitro (Fig. 2B) displayed the wt phenotype in all transfection assays (Fig. 4 and 5), as was the case for the original integrase mutant (Fig. 1) (2). The integrase mutant virus obtained by *trans* complementation (Fig. 3), however, was unable to express the marker CAT gene after infection of CD4-positive target cells in a single-step replication system (Fig. 6). Although the conclusion described above represents a sharp contrast to that of a previous report (25), it is consistent with the data obtained with the other retroviruses (6, 14, 19, 20). If integration is not required for efficient expression of viral genes, as claimed in a previous article (25), one must assume that the integrase has some functional role in the late infection phase because all integrase mutants described so far are unable to establish a productive infection. Our data in

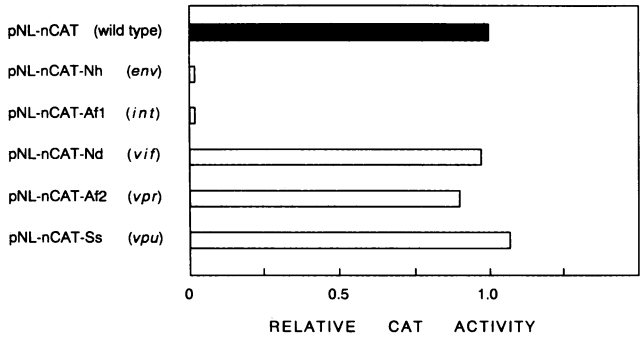

FIG. 6. Analysis of early phase of viral replication by a single-round infection assay. Virus samples obtained from cotransfections of pNL-nCAT constructs and prev1 (Fig. 3) were inoculated into CD4-positive A3.01 cells, and 48 h later, CAT activity in the cell lysates was monitored. Relative activity is shown. CAT activity detected in the cells infected with the wt NLnCAT virus was 28.2% (conversion ratio). Mutant designations and mutated genes (in parentheses) are indicated.

Fig. 1, 5, and 6 do not support this hypothesis. The molecular basis for requirement of integration to efficiently express viral genes remains to be investigated.

Another important finding in this report is that the HIV-1 *vif*, *vpr*, and *vpu* proteins appear not to have an important role in the early infection phase (Fig. 6). As for the *vpu* and *vpr* proteins, this result was well expected. The proviral *vpu* mutant was reported to be defective in the very late stage of viral replication cycle (16). The growth kinetics of the *vpr* mutant viruses in CD4-positive target cells were only slightly delayed relative to those of wt virus (17), and it would be difficult to detect the mutational effect. In contrast to these two proteins, the *vif* protein was postulated to be required in the early phase of HIV-1 infection in CD4-positive cells (26).

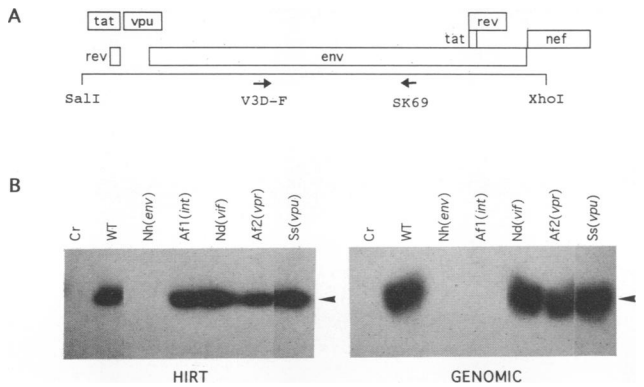

FIG. 7. PCR analysis of genomic and Hirt DNAs prepared from A3.01 cells infected with various viruses. (A) Location of the primers V3D-F and SK69 within an *env* gene. Location of the restriction sites (*Sal*I and *Xho*I) used to cut out a fragment for a ^{32}P-labeled probe (used for panel B) is also indicated. (B) Southern blot analysis of the amplified products by PCR. A3.01 cells were infected with virus as described in the legend to Fig. 1, and DNA was extracted from infected cells for PCR amplification at the appropriate time (see Materials and Methods). The PCR products were run through a 3% agarose gel and analyzed by Southern hybridization. The results obtained with DNA samples from extrachromosomal and genomic fractions are shown in panels HIRT and GENOMIC, respectively. Arrowheads indicate the size (937 bp) of amplified products predicted from the location of the primers.

The *vif* mutant virus obtained from transfected SW480 cells was normal with respect to infectivity as judged by a single-step infection assay (Fig. 6). This result showed that the *vif* protein is not required in the early infection stage. A more detailed study is necessary to precisely determine the functional role of the HIV-1 *vif* protein.

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